MOLECULAR PHARMACOLOGY, 21:564–569

The Opiate Receptor-Binding Interactions of Opiate Alkaloids and of an Opioid Peptide in Rat Brain Membranes

Selection by Manganese Ions and by Cholic Acid (Sodium Salt) and Minimalization of Cross-Reaction *in Vitro*

Y. KOUAKOU, J. M. ZAJAC, C. MOISAND, AND J. C. MEUNIER

Laboratoire de Pharmacologie et de Toxicologie Fondamentales, Centre National de la Recherche Scientifique, 31400 Toulouse, France

Received November 9, 1981; Accepted January 21, 1982

SUMMARY

We have analyzed the effects of cations (Na^+, K^+, Mg^{2+}, Mn^{2+}, Ca^{2+}) and of detergents (Triton X-100, sodium cholate) on the binding of two opiate alkaloids ([^{3}H]morphine, [^{3}H]dihydromorphine (DHM)) and of one opioid peptide, D-Ala^2-[tyrosyl-3,5-^{3}H]enkephalin (5-D-leucine), in rat brain membranes. When a fixed, reasonably low concentration of each radioligand is used so that each would predominantly label its high-affinity site, we observe that (a) monovalent cations, Na^+ in particular, inhibit nearly equally well the binding of the μ [morphine, dihydromorphine] and δ [D-Ala^2, D-Leu^5-enkephalin (DADL)] agonists. (b) Binding of the μ and δ agonists is differentially affected by divalent cations. Binding of morphine and DHM is markedly inhibited in a monophasic manner by Mn^{2+} [$I_{50} \sim 4$ mM). In contrast, a clearly biphasic effect on binding of DADL, the δ agonist, is found: enhanced binding at millimolar concentrations of Mn^{2+} followed by inhibition ($I_{50} \sim 40$ mM). (c) *Conversely*, binding of DADL is more readily inhibited in the presence of nonsolubilizing concentrations (<0.1%, w:v) of cholic acid (sodium salt) than is binding of the μ agonists. In saturation experiments, ^{3}H-DADL is found to interact with a high- and a low-affinity site in our membrane preparation. It is shown that Mn^{2+} (10–20 mM) selectively inhibits binding of the tritium-labeled pentapeptide to the *low*-affinity site, whereas sodium cholate (0.05–0.1%, w:v) selectively prevents binding of the radioligand to the *high*-affinity site. These results emphasize the importance of the environment in evaluating ligand-receptor interactions and suggest novel experimental conditions for an assay of μ and δ receptor subtypes with minimal cross-reaction in nerve tissue.

INTRODUCTION

In 1976, using morphine and benzomorphans in chronically spinal-injured dogs, Martin *et al.* (1) observed a variety of pharmacological responses that led them to postulate the existence of three types of opiate receptors: *mu*, *kappa*, and *sigma*. Since then, evidence has accumulated (2) that brain membranes contain at least two opiate receptor subtypes, μ (morphine) and δ (enkephalin) whose properties do not correspond well to the definition of Martin *et al.* (1).

Receptors of the μ type may mediate analgesia, whereas δ receptors have been implicated in other effects, including behavioral effects (3–7). On the basis of biochemical binding studies in rat brain membranes, Chang *et al.* (8) have classified opiates and enkephalins into seven categories, elegantly pointing out that "it is the relative occupancy of μ and δ receptors by agonists and antagonists that determines the specific pharmacological profile of a given drug at a particular dose." However, Gillan *et al.* (9), after measuring the binding capacities of various tritiated ligands in guinea pig brain, concluded that sites other than μ and δ may have been present in their preparations. They speculated that these sites correspond to the κ receptor of Martin *et al.* (1), a speculation that is supported by recent data (10, 11).

Several peptides with opiate-like properties have been characterized in nerve tissue. These include Met- and Leu-enkephalin (12), β-endorphin (13), dynorphin (14), and Met-enkephalin-Arg^6-Phe^7 (15). To date, it remains to be proven whether all of these peptides are biologically significant and whether any one serves as a physiological agonist of any one of the three receptor subtypes in brain.

Structural and functional characterization of these multiple receptors requires that they be probed selectively. This may be achieved by selecting either the adequate effector or the appropriate target (binding site),

0026-895X/82/030564-06$02.00/0

or both. Along these lines, new peptides have been synthesized which display enhanced specificity toward either δ sites [Tyr-D-Ser-Gly-Phe-Leu-Thr (16, 17)] or μ sites [Tyr-Pro-Phe-Pro, "morphiceptin" (18), and Tyr-D-Ala-Gly-MePheNH$(CH_2)_2$OH, "RX783006" (19)]. On the other hand, opiate binding in nerve tissue has long been known to be affected by a number of agents, including ions (20, 21), GTP (22), and *N*-ethylmaleimide (23).

In the present article we have searched for environmental conditions selective of either μ or δ opiate binding interactions *in vitro*. Definition of the conditions favorable for minimal cross-reaction of δ agonists with receptor subtype μ and vice versa follows from the observations that (a) unlike monovalent cations, divalent cations, Mn^{2+} in particular, are substantially better inhibitors of μ than of δ agonist binding; (b) binding of δ agonists is much more sensitive to sodium cholate than is binding of μ agonists. These results define new experimental conditions for a quantitative biochemical binding assay of μ and δ receptor sites *in vitro*, using current available radioligands.

MATERIALS AND METHODS

Preparation of the CMF[1] *from rat brain.* Adult Wistar rats of either sex were killed by decapitation. Their brains were excised and the cerebella were discarded. The freshly dissected, weighed tissue, in a final volume of 1.2 ml (per 100 mg) of 0.32 M sucrose in Tris-HCl (1 mM, pH 7.4), was homogenized at 4° in a Potter-Elvehjem tissue grinder (10 strokes; 800–1000 rpm).

The membrane suspension was incubated for 30 min at 35° and centrifuged (0–2°) in a Beckman rotor (Type 30) for 30 min at 30,000 rpm. The supernatant was discarded, and the pellet was dispersed (Polytron) in a large excess of ice-cold Tris-HCl (50 mM, pH 7.4) and centrifuged again as before. The resulting, washed pellet was homogenized (Polytron) in the original volume of buffer to yield the CMF (6–7 mg of protein per milliliter) used in these experiments.

Biochemical binding studies. Binding studies were carried out at 25° or 35° in Tris-HCl (50 mM, pH 7.4). Each assay mixture (1 ml, in triplicate or sextuplate) contained 0.30–0.35 mg of CMF protein, the radioligand at the desired concentration with and without 10 μM levorphanol. After incubation (60 min at 25° or 30 min at 35°), the reaction was "stopped" by rapid cooling (melting ice bath) and immediate filtering through glass-fiber disks (Whatman GF/B) on Millipore Model 1225 sampling manifolds. Unbound or loosely bound radioactivity was washed away with two 5-ml portions of ice-cold buffer. Filtering and washing 48 samples required no more than 5.5 min. The filters were dried and counted for radioactivity in 4 ml of Beckman Ready-Solv EP cocktail by a Kontron Model MR 300 automatic liquid scintillation system.

Radioligands. Morphine, [*N-methyl*-^{3}H] (40–60 Ci/mmole), and dihydromorphine, [*N-methyl*-^{3}H] (70–90 Ci/mmole) were obtained from New England Nuclear Corporation (Dreieich, West Germany). D-Ala^2-[*tyrosyl*-3,5-^{3}H]Enkephalin (5-D-leucine) (15–40 Ci/mmole) was obtained from the Radiochemical Center (Amersham, England).

RESULTS

Cross-reaction: theoretical and practical considerations. In the simplest case of a ligand L that interacts reversibly with two receptor subtypes R_1 and R_2, the following equilibria are to be considered:

$$R_1 + L \leftrightarrows R_1L$$

$$R_2 + L \leftrightarrows R_2L$$

According to the law of mass action,

$$[R_1L] = [R_1]_t \times [L] \times (K_{d1} + [L])^{-1}$$

$$[R_2L] = [R_2]_t \times [L] \times (K_{d2} + [L])^{-1}$$

where $[R_1L]$ and $[R_2L]$ are the concentrations of sites R_1 and R_2 occupied by L, $[L]$ is the concentration of free ligand, and K_{d1} and K_{d2} are the equilibrium dissociation constants of complexes R_1L and R_2L, respectively. $[R_1]_t$ and $[R_2]_t$ represent the total concentrations of sites R_1 and R_2.

Ligand L is referred to as Type 1 if $K_{d1} < K_{d2}$, as Type 2 if $K_{d1} > K_{d2}$, and as Type 1,2 if $K_{d1} = K_{d2}$.

Cross-reaction refers to the interaction (usually undesired) between a ligand of Type 1 and R_2 or between a ligand of Type 2 and R_1. In the case of a ligand of Type 1, cross-reaction is defined as the fraction b_2/b_t of bound L associated with R_2, i.e.,

$$\frac{b_2}{b_t} = \frac{[R_2L]}{[R_1L] + [R_2L]} = \left(1 + \frac{[R_1]_t}{[R_2]_t} \times \frac{K_{d2} + [L]}{K_{d1} + [L]}\right)^{-1}$$

Since $[L] \in (0, \infty)$, then

$$\left(1 + \frac{[R_1]_t}{[R_2]_t} \times \frac{K_{d2}}{K_{d1}}\right)^{-1} \leq \frac{b_2}{b_t} < \left(1 + \frac{[R_1]_t}{[R_2]_t}\right)^{-1}$$

Therefore, the *minimal* cross-reaction is a hyperbolic function of the product of two ratios, an affinity ratio (characteristic of the ligand) and a capacity ratio (characteristic of the preparation). The maximal cross-reaction depends only on the capacity ratio and decreases hyperbolically when the latter increases.

In light of these results, it may be of interest to calculate the limits of b_2/b_t in the case of opiate alkaloids and of opioid peptides commonly used in binding and autoradiographic studies. Four ligands among the very few for which K_{d1} and K_{d2} values have been reported (2) will be tested: morphine and naloxone (Type 1), DADL (Type 2), and etorphine (Types 1 and 2). Table 1 gives the calculated values of minimal and maximal cross-reaction of these ligands at their low-affinity site in membrane preparations from rat whole brain, thalamus, and frontal cortex.

Owing to their high affinity ratios, 111 and 26, respectively, morphine and naloxone would exhibit negligible *minimal* cross-reaction with receptor subtype 2 (δ) in all regions considered, including frontal cortex (0.63 and 2.5%, respectively). Therefore, provided that they are used at very low concentration ([L] ~ 0), morphine and

[1] The abbreviations used are: CMF, crude membrane fraction; DHM, dihydromorphine; DADL, D-Ala^2,D-Leu^5-enkephalin.

TABLE 1

Calculated cross-reaction (minimal and maximal) of various opioid ligands with opiate receptor subtypes 1 (μ) and 2 (δ) in three membrane preparations from rat brain

Except for etorphine, the values (dissociation constants and capacity ratios) introduced in the calculations are those reported by Chang and Cuatrecasas (2) and by Chang *et al.* (11). The contribution of opiate receptor subtype 3 (benzomorphans) has been omitted.

Type	Ligand	K_{d1}	K_{d2}	Minimal (maximal) cross-reaction with low-affinity site		
				Whole brain (R_1/R_2 = 2.0)	Thalamus (R_1/R_2 = 9.9)	Frontal cortex (R_1/R_2 = 1.5)
		nM	*nM*	*% of total*		
1	Morphine	0.3	32	0.47 (33)	0.095 (9.2)	0.63 (40)
	Naloxone	0.5	13	1.9 (33)	0.39 (9.2)	2.5 (40)
2	DADL	6.0	0.8	21 (67)	57 (91)	17 (60)
1,2	Etorphine					
	As 1	0.14	0.14	33 (33)	9.2 (9.2)	40 (40)
	As 2	0.14	0.14	67 (67)	91 (91)	60 (60)

naloxone may be termed "selective probe of receptor subtype 1" (μ).

In contrast, DADL, whose affinity ratio is reported to be 0.13 (2), displays substantial *minimal* cross-reaction with receptor subtype 1 (μ), even in frontal cortex (17%). In thalamus, the pentapeptide would label predominantly subtype 1 even when used at very low concentration. Therefore DADL should not be considered as the "selective probe of receptor subtype 2" (δ), at least when tested under standard assay conditions.

Monovalent cations inhibit nearly equally well binding of μ and δ opiate agonists in rat brain membranes. We have analyzed the effect of sodium, potassium, and choline chlorides (5–200 mM) on the binding of [^{3}H] morphine (1.0 nM), [^{3}H]DHM (0.5 nM), and ^{3}H-DADL (2.0 nM). Choline chloride had little if any effect, indicating little if any contribution of ionic strength to the phenomenon. Sodium chloride inhibited binding of all ligands in rat brain CMF. The inhibition curves were roughly parallel, yielding parallel Hill representations of slopes near unity (Fig. 1). The concentrations of Na^+ required to halve binding of the three ligands were 30 (DHM), 40 (morphine), and 60 mM (DADL). Potassium exerted a very similar inhibitory action except that higher concentrations were necessary (data not shown). Therefore it was concluded that the opiate receptor binding interactions of μ and δ opiate agonists were poorly discriminated by monovalent cations, in particular Na^+, in our membrane preparation.

Divalent cations preferentially inhibit binding of μ opiate agonists in rat brain membranes. With divalent cations, the situation was entirely different. Binding of the μ agonists, morphine and DHM, was markedly inhibited in the presence of Mg^{2+}, Mn^{2+}, or Ca^{2+} (0.5–70 mM). For instance, a concentration as low as 4 mM $MnCl_2$ reduced binding of [^{3}H]morphine and of [^{3}H]DHM to 50% of the control value in rat CMF (Fig. 2).

Binding of the δ agonist was affected in a more complicated manner. In the presence of millimolar concentrations of Mn^{2+}, there was a significant increase (30–60%) of specifically bound ^{3}H-DADL. This increase, bell-shaped, was followed by an inhibitory phase, 50%

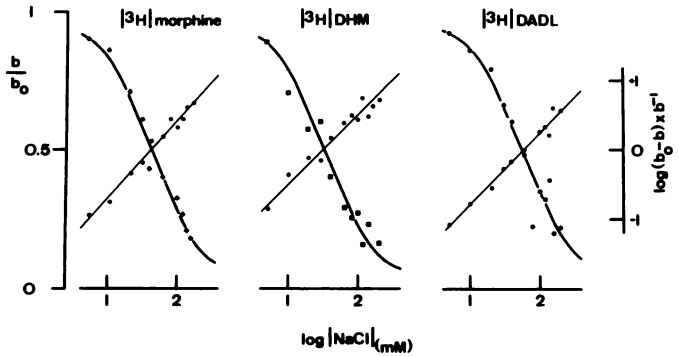

FIG. 1. *Inhibition by sodium ions of the binding of morphine, DHM, and DADL in rat brain membranes*

The radioligands were used at 1.0 nM ([^{3}H]morphine), 0.5 nM ([^{3}H] DHM), and 2.0 nM (^{3}H-DADL). To avoid confusion, the inhibition curves are drawn separately. The corresponding Hill representations (O) are also shown. Each experimental value, expressed as percentage of control (in sextuplet) is the mean of triplicate determinations.

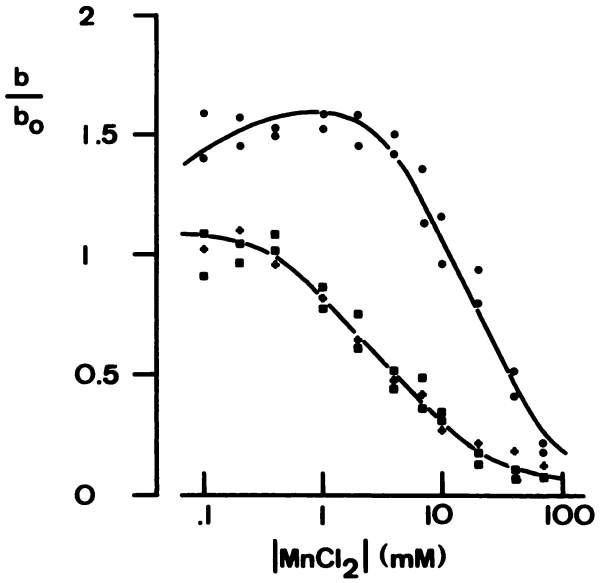

FIG. 2. *Effect of manganese ions on the binding of morphine, DHM, and DADL in rat brain membranes*

The radioligands were at the concentrations indicated in the legend to Fig. 1. +, [^{3}H]Morphine (one experiment); ■, [^{3}H]DHM (two independent experiments); ●, ^{3}H-DADL (two independent experiments). Each experimental value, expressed as percentage of control (in sextuplet) is the mean of triplicate determinations.

inhibition of ^{3}H-DADL binding being recorded at 40 mM Mn^{2+}.

The two other divalent cations, Mg^{2+} and Ca^{2+}, had effects very similar (at least qualitatively) to those elicited by manganese. These results demonstrate clear differentiation between μ and δ opiate-receptor interactions by *divalent* cations in brain membranes. Enhanced binding (increased affinity?) in the presence of low concentrations (~1 mM) of these ions appears to be inherent to δ sites in nerve tissue (see Discussion). However, in practice it was more important to note that binding of ^{3}H-DADL was unaffected in the presence of 10–20 mM Mn^{2+} whereas binding of [^{3}H]morphine and of [^{3}H]DHM under these conditions was decreased by as much as 80% (Fig. 2).

Cholic acid (sodium salt) preferentially inhibits binding of the δ opiate agonist in rat brain membranes. In addition to cations, two detergents were tried: Triton X-100 and cholic acid (sodium salt). At nonsolubilizing concentrations (<0.1%, w:v), these agents were known[2] to inhibit binding of [^{3}H]etorphine and of [^{3}H]diprenorphine in our membrane preparations. However, since oripavine derivatives are specific for neither μ nor δ sites (24), it was necessary to repeat these experiments with more selective radioligands.

Binding of the μ and δ agonists was extremely sensitive to Triton X-100. In fact, binding of the three radioligands was halved in the presence of concentrations of Triton X-100 as low as 0.001–0.004% (w:v). Binding of ^{3}H-DADL appeared to be hardly more sensitive to this detergent than did binding of the two alkaloids (data not shown).

With sodium cholate, clear differences were observed (Fig. 3). Binding of the μ agonists was only moderately inhibited (<20%) by the bile salt at concentrations in the range 0.005–0.1% (w:v). Over this same range, sodium cholate exerted monotonous and almost complete inhibition of ^{3}H-DADL binding, 50% being reached at 0.03–0.04% (w:v).

Therefore cholic acid (sodium salt), unlike Triton X-100, preferentially inhibited binding of the δ opiate agonist in rat brain CMF.

Washing these membranes free of sodium cholate (by centrifugation) resulted in increased (50%) binding of the three radioligands. It is not known whether the phenomenon rests on increased affinity following removal of tightly bound endogenous ligands upon incubation with detergent. This particular problem is currently being investigated.

Manganese chloride and sodium cholate respectively select opiate receptor subtype δ and opiate receptor subtype μ in rat brain membranes. In rat brain CMF, saturation experiments using ^{3}H-DADL at concentrations in the range 0.4–20 nM suggested interaction of this radioligand with at least two distinct classes of binding sites. The Scatchard representations were clearly biphasic (Fig. 4a and b): one-third (0.1 pmole/mg of protein) of the sites exhibited high affinity (~1.3 nM) for the pentapeptide and were likely to represent δ receptor sites. The other two-thirds (0.2 pmole/mg) displayed somewhat lower affinity (10–15 nM) and could represent

[2] J. M. Zajac and J. C. Meunier, unpublished observation.

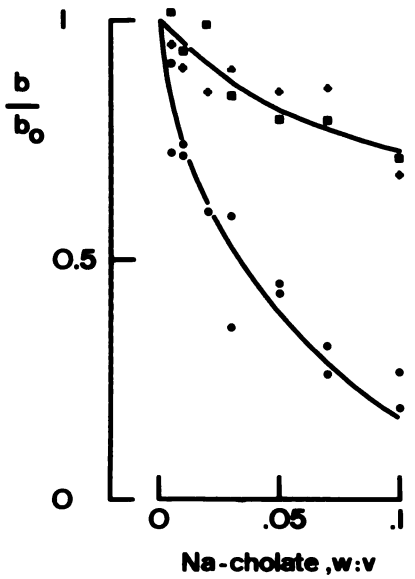

FIG. 3. *Effect of cholic acid (sodium salt) on the binding of morphine, DHM, and DADL in rat brain membranes*

Conditions and symbols are the same as in the legends to Figs. 1 and 2.

receptor subtype μ. These results agree well with those obtained in a previous study (17).

Figure 4a shows that saturation experiments conducted in the presence of 10 mM $MnCl_2$ resulted in significant linearization of Scatchard representations with a selective loss of the pentapeptide's low-affinity site. At 20 mM $MnCl_2$, the effect was even more pronounced, with a complete disappearance of the DADL *low*-affinity site. The high (δ)-affinity site (0.09 pmole/

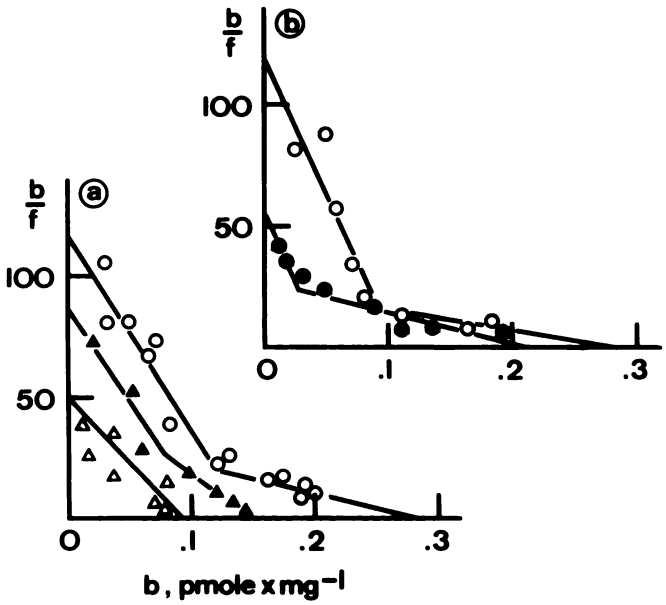

FIG. 4. *Scatchard analysis of the binding of ^{3}H-DADL in rat brain membranes: the effects of manganese chloride and sodium cholate*

○, Control experiments in 50 mM Tris-HCl (pH 7.4) (no additions). a, The effect of manganese ions present at 10 mM (▲) and at 20 mM (△); the pentapeptide's low-affinity binding site is selectively lost. b, It is evident that sodium cholate (0.07%, w:v; ●) specifically inhibits binding of ^{3}H-DADL to the high-affinity site.

mg) was unaffected except perhaps for a slight decrease (2-fold) of affinity.

When cholic acid (sodium salt) was used instead of $MnCl_2$, the effect was exactly opposite: the pentapeptide's *high*-affinity site was preferentially lost (Fig. 4b).

These experiments demonstrated clearly the feasibility of "occluding" selectively either opiate receptor subtype μ (with manganese ions) or opiate receptor subtype δ (with sodium cholate).

DISCUSSION

On the basis of simple hypotheses and with the use of published data, we calculated that morphine and naloxone, owing to their high affinity ratios (111 and 26, respectively) would label receptor subtype μ with nearly absolute selectivity when used at $[L] \sim 0$. The two alkaloids are routinely available at high specific radioactivity (40–60 Ci/mmole). Therefore, it is puzzling why Sandoz peptide FK-33824, whose affinity ratio is only one-tenth that of morphine (25), is so often used in place of morphine as the specific μ probe in biochemical binding (25) and autoradiographic (26) studies.

There is some agreement that the affinity ratio of DADL, the δ agonist, does not exceed 10 (17) and may be as low as 2.7 (26). Consequently, the pentapeptide can hardly be used as a selective δ probe, especially in those regions of the brain in which subtype μ largely predominates. In rat thalamus, a region which, according to Chang *et al.* (11), is considerably enriched in μ sites, DADL would display a *minimal* cross-reaction of 57%. In other words, ^{3}H-DADL bound in membrane preparations from this particular region would reveal μ rather than δ sites at any concentration of radioligand.

In the present article we report data on the differential "regulation" (inhibition) by manganese ions and cholic acid (sodium salt) of the binding of μ and δ agonists in rat brain membranes.

Initially, ionic effects on opiate (usually alkaloids) binding in nerve tissue were interpreted exclusively in terms of reversible agonist-antagonist receptor transconformations (20). Later, it was generally accepted that binding of μ agonists was more stringently regulated by sodium ions that bound δ agonists (27). This is not the case. Monovalent cations, sodium in particular, inhibit μ and δ binding interactions nearly equally well in brain membranes. Since δ antagonists are not available, the mechanism whereby δ-opiate receptor interactions are sodium-inhibited remains to be elucidated.

Divalent cations were previously shown to enhance binding of opiate (usually alkaloids) agonists to their receptor sites (21). However, when low concentrations of μ (morphine, DHM) and δ (DADL) agonists are used, it becomes evident that Mn^{2+} differentially affects binding of the two sets of radioligands.

Enhanced binding in the presence of millimolar concentrations of Mn^{2+} appears to be characteristic of the δ agonist (2). Pertaining to this notion is the established fact that, in brain membranes, binding of leucine enkephalin is markedly increased by Mn^{2+} whereas binding of methionine enkephalin is not (28). Taken together, these data would support the proposal of Snyder and Goodman (27) that leucine enkephalin serves as a physiological agonist of receptor subtype δ in nerve tissue. This hypothesis is being tested in rabbit cerebellum, which, in addition to being rich in [^{3}H]etorphine binding sites (29), contains substantial quantities of methionine enkephalin but virtually no leucine enkephalin (30).

Manganese ions were also shown here to inhibit binding of ^{3}H-DADL but at concentrations 10-fold higher than those required to inhibit binding of [^{3}H]morphine and [^{3}H]DHM. A concentration of 10–20 mM $MnCl_2$ caused no inhibition of binding of the δ agonist but decreased that of the μ agonists by as much as 80%. In addition, saturation experiments carried out with ^{3}H-DADL indicated that 10–20 mM Mn^{2+} had little if any effect on the pentapeptide's high-affinity binding (δ interaction) but markedly inhibited its low-affinity binding (μ interaction). Therefore, experimental conditions were met which were aimed at minimalizing cross-reaction of the δ agonist with receptor subtype μ *in vitro*.

Finally, our finding that binding of ^{3}H-DADL was more sensitive to cholic acid (sodium salt) than was binding of [^{3}H]morphine and [^{3}H]DHM provided a complementary mean of discriminating between δ and μ receptor interactions *in vitro*. This proposition was clearly supported by the demonstration of a selective loss of the pentapeptide's high-affinity (δ) site in the presence of nonsolubilizing concentrations of the anionic detergent.

As we have mentioned before (see Introduction), the specific labeling of a particular opiate receptor subtype may be achieved by selecting either the adequate effector or the appropriate target (binding site). The present study has demonstrated the feasibility of the second approach with natural emphasis on receptor subtype δ.

In conclusion and briefly stated, the results that we have reported here (a) provide additional evidence for multiple opiate receptors in brain, (b) reaffirm the importance of the environment in evaluating ligand-receptor interactions, and (c) suggest novel experimental conditions for an assay of μ and δ opiate receptor sites with minimal cross-reaction in nerve tissue.

Addendum. While this article was being reviewed, Hiller *et al.* (31) reported the selective inhibition of binding to δ receptors by alcohol. Alcohol and cholate (this study) may act through a common mechanism at the membrane level.

REFERENCES

1. Martin, W. R., C. G. Eades, J. A. Thompson, R. E. Huppler, and P. E. Gilbert. The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* **197**:517–522 (1976).
2. Chang, K. J., and P. Cuatrecasas. Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* **254**:2610–2618 (1979).
3. Urca, G., H. Frenk, J. C. Liebeskind, and A. N. Taylor. Morphine and enkephalin: analgesic and epileptic properties. (*Wash. D. C.*) *Science* **197**:83–86 (1977).
4. Herz, A., J. Bläsig, H. M. Emrich, C. Cording, S. Pirée, A. Kölling, and D. V. Zerssen. Is there some indication from behavioral effects of endorphins for their involvement in psychiatric disorders? *Adv. Biochem. Pharmacol.* **18**:333–339 (1978).
5. Frenk, H., G. Urca, and J. C. Liebeskind. Epileptic properties of leucine- and methionine-enkephalin: comparison with morphine and reversibility with naloxone. *Brain Res.* **147**:327–337 (1978).
6. Stern, L., and J. D. Belluzi. Brain endorphins: possible role in reward and memory formation. *Fed. Proc.* **38**:2468–2472 (1979).
7. Lewis, E. L., M. Mishkin, E. Bragin, R. M. Brown, C. B. Pert, and A. Pert.

Opiate receptor gradients in monkey cerebral cortex: correspondence with sensory processing hierarchies. *Science* (*Wash. D. C.*) **211**:1166–1169 (1981).
8. Chang, K. J., E. Hazum, and P. Cuatrecasas. Possible role of distinct morphine and enkephalin receptors in mediating actions of benzomorphan drugs (putative kappa and sigma agonists). *Proc. Natl. Acad. Sci. U. S. A.* **77**:4469–4473 (1980).
9. Gillan, M. G. C., H. W. Kosterlitz, and S. J. Paterson. Comparison of the binding characteristics of tritiated opiates and opioid peptides. *Br. J. Pharmacol.* **70**:481–490 (1980).
10. Kosterlitz, H. W., S. J. Paterson, and L. E. Robson. Characterization of the kappa subtype of the opiate receptor in the guinea-pig brain. *Br. J. Pharmacol.* **73**:939–949 (1981).
11. Chang, K. J., E. Hazum, and P. Cuatrecasas. Novel opiate binding sites selective for benzomorphan drugs. *Proc. Natl. Acad. Sci. U. S. A.* **78**: 4141–4145 (1981).
12. Hughes, J., T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, and H. R. Morris. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* (*Lond.*) **258**:577–579 (1975).
13. Li, C. H., and D. Chung. Isolation of an untriakontapeptide with opiate activity from camel pituitary glands. *Proc. Natl. Acad. Sci. U. S. A.* **73**:1145–1148 (1976).
14. Goldstein, A., S. Tachibana, L. I. Lowney, M. Hunkapiller, and L. Hood. Dynorphin-(1–13), an extraordinarily potent opioid peptide. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6666–6670 (1979).
15. Stern, A. S., R. W. Lewis, S. Kimura, J. Rossier, L. D. Gerber, L. Brink, S. Stein, and S. Udenfriend. Isolation of the opioid heptapeptide Met-enkephalin [Arg^6,Phe^7] from bovine adrenal medullary granules and striatum. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6680–6683 (1979).
16. Gacel, G., M. C. Fournié-Zaluski, and B. P. Roques. Tyr-D-Ser-Gly-Phe-Leu-Thr, a highly preferential ligand for δ-opiate receptors. *F. E. B. S. Lett.* **118**:245–247 (1980).
17. David, M., C. Moisand, J. C. Meunier, J. L. Morgat, G. Gacel, and B. P. Roques. [^{3}H]Tyr-D-Ser-Gly-Phe-Leu-Thr: a selective probe for the δ-opiate receptor subtype in brain. *Eur. J. Pharmacol.*, in press.
18. Chang, K. J., A. Killian, E. Hazum, P. Cuatrecasas, and J. K. Chang. Morphiceptin (NH_4-Tyr-Pro-Phe-Pro-$CONH_2$): a potent and specific agonist for morphine (μ) receptors. *Science* (*Wash. D. C.*) **212**:75–77 (1981).
19. Handa, B. K., A. C. Lane, J. A. H. Lord, B. A. Morgan, M. J. Rance, and C. F. C. Smith. Analogues of β-LPH_{61-64} possessing selective agonist activity at μ-opiate receptors. *Eur. J. Pharmacol.* **70**:531–540 (1981).
20. Pert, C. B., G. Pasternak, and S. H. Snyder. Opiate agonists and antagonists discriminated by receptor binding in brain. *Science* (*Wash. D. C.*) **182**:1359–1361 (1973).
21. Pasternak, G. W., A. Snowman, and S. H. Snyder. Selective enhancement of [^{3}H]opiate agonist binding by divalent cations. *Mol. Pharmacol.* **11**:735–744 (1975).
22. Blume, A. J. Interaction of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1713–1717 (1978).
23. Simon, E. J. and J. Groth. Kinetics of opiate receptor inactivation by sulfhydryl reagents: evidence for conformational change in presence of sodium ions. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2404–2407 (1975).
24. Kosterlitz, H. W., and S. J. Paterson. Characterization of opioid receptors in nervous tissue. *Proc. R. Soc. Lond. B Biol. Sci.* **210**:113–122 (1980).
25. Chang, K. J., B. R. Cooper, E. Hazum, and P. Cuatrecasas. Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. *Mol. Pharmacol.* **16**:91–104 (1979).
26. Goodman, R. R., S. H. Snyder, M. J. Kuhar, and W. S. Young. Differentiation of delta and mu opiate receptor localization by light microscopic autoradiography. *Proc. Natl. Acad. Sci. U. S. A.* **77**:6239–6243 (1980).
27. Snyder, S. H., and R. R. Goodman. Multiple neurotransmitter receptors. *J. Neurochem.* **35**:5–15 (1980).
28. Meunier, J. C., and C. Moisand. Binding of Leu^5-enkephalin and Met^5-enkephalin to a particulate fraction from rat cerebrum. *F. E. B. S. Lett.* **77**:209–213 (1977).
29. Meunier, J. C., and J. M. Zajac. Cerebellar opiate receptors in lagomorphs. Demonstration, characterization and regional distribution. *Brain Res.* **168**:311–321 (1979).
30. Hughes, J., H. W. Kosterlitz, and T. W. Smith. The distribution of methionine-enkephalin and leucine-enkephalin in the brain and periphenal tissues. *Br. J. Pharmacol.* **61**:639–647 (1977).
31. Hiller, J. M., L. M. Angel, and E. J. Simon. Multiple opiate receptors: alcohol selectively inhibits binding to delta receptors. *Science* (*Wash. D. C.*) **214**:468–469 (1981).

Send reprint requests to: Dr. J. C. Meunier, Laboratoire de Pharmacologie et de Toxicologie Fondamentales, Centre National de la Recherche Scientifique, 205 route de Narbonne, 31400 Toulouse, France.